Scand. J. Immunol. **54**, 448–452, 2001

Immunological Characterization of Antigens Encoded by the RD1 Region of the *Mycobacterium tuberculosis* Genome

P. N. BRUSASCA,* R. COLANGELI,* K. P. LYASHCHENKO,* X. ZHAO,* M. VOGELSTEIN,* J. S. SPENCER,† D. N. McMURRAY‡ & M. L. GENNARO*

**Public Health Research Institute, New York, NY 10016; †Colorado State University, Fort Collins, CO 80523; ‡Texas A & M University System Health Science Center, College Station, TX 77843, USA*

(Received 19 October 2000; Accepted in revised form 21 May 2001)

Brusasca PN, Colangeli R, Lyashchenko KP, Zhao X, Vogelstein M, Spencer JS, McMurray DN, Gennaro ML. Immunological Characterization of Antigens Encoded by the RD1 Region of the *Mycobacterium tuberculosis* Genome. Scand J Immunol 2001;54:448–452

Development of immunoassays specific for the diagnosis of tuberculosis requires antigens unique to *Mycobacterium tuberculosis*. In a search for such antigens we tested six proteins encoded by RD1, a region present in *M. tuberculosis* and virulent *M. bovis* genomes but missing from the DNA of all substrains of *M. bovis* Bacillus Calmette-Guerin (BCG). The six proteins (Rv3871, Rv3872, Rv3873, MTSA-10, ESAT-6 and Rv3878) were purified to near-homogeneity from recombinant *Escherichia coli*. When tested for the ability to elicit antibody responses and delayed type hypersensitivity in tuberculous guinea pigs, only two of six antigens, ESAT-6 and MTSA-10, elicited strong skin reactions, while vigorous antibody responses were observed to all six proteins. When antibody responses to RD1 antigens were evaluated in sera from patients having pulmonary tuberculosis and from control subjects (patients having mycobacterioses other than tuberculosis, and healthy persons), a sizeable proportion (25%) of tuberculosis patients but none of the control subjects, had antibodies against MTSA-10 and/or ESAT-6. We conclude that MTSA-10 and ESAT-6 are promising candidates for immunodiagnostic assays specific for tuberculosis.

Dr M. L. Gennaro, Public Health Research Institute, New York, NY 10016, USA. E-mail: gennaro@phri.nyu.edu

INTRODUCTION

Advances in tuberculosis (TB) control require new, improved tools for the diagnosis of tuberculous infection and disease (reviewed in [1,2]). Immunological assays, which have been prominent in TB diagnosis ever since the discovery of tuberculin by Robert Koch in the 1890's, need to fulfil two requirements for improved accuracy. First, antigens in the tests must be specific for *Mycobacterium tuberculosis* to avoid false-positive test results with subjects vaccinated with *M. bovis* BCG and with persons living in areas having a high load of environmental mycobacteria. Secondly, cocktails of multiple antigens must be formulated to cover the heterogeneous antigen recognition that is characteristic of TB [3,4].

The search for antigens specific for *M. tuberculosis* is now greatly facilitated by our knowledge of the genome sequence of *M. tuberculosis* [5] and the on-going sequence analyses of genomes of other, tuberculous and nontuberculous, mycobacteria (http://www.sanger.ac.uk/Projects/; http://www.tigr.org/tdb/). Thanks to comparative genomics (for examples see [6, 7]), bioinformatic analyses can readily identify genes that are absent from the DNA of *M. bovis* BCG substrains and that lack homologs in nontuberculous mycobacteria most commonly associated with human disease, such as *M. avium* [8]. In the present report, we have performed immunological characterization of proteins encoded by RD1, a region present in the *M. tuberculosis* and virulent *M. bovis* genomes but absent from the DNA of all substrains of *M. bovis* BCG [9].

MATERIALS AND METHODS

Antigens. Genes located in the RD1 region were amplified from DNA of *M. tuberculosis* $H_{37}Rv$ by polymerase chain reaction (PCR) and cloned into the *Escherichia coli* vector pQE-30 (Qiagen) following standard protocols [10]. Recombinant proteins were purified to

near-homogeneity from cultures of *E. coli* as polyhistidine-tagged fusion proteins by using a published three-step purification protocol [11]. The protocol usually yields protein preparations that are > 99% pure from protein contaminants and that have a content of lipopolysaccharide lower than 10 pg/μg of protein [11], a level that is below the sensitivity of guinea pig skin tests [12, 13].

Guinea pig sera. Sera were obtained at 15 weeks postinfection from 14 random-bred, Hartley strain guinea pigs (Charles River Breeding Laboratories, Wilmington, MA, USA) exposed to 10–15 colony-forming units (CFU) of *M. tuberculosis* $H_{37}Rv$ per animal in an aerosol chamber designed to deliver droplet nuclei directly into the alveolar spaces [14]. Control sera were obtained from nine uninfected animals.

Human sera. Sera were obtained from 75 patients having pulmonary TB, 16 patients having mycobacterioses other than tuberculosis (MOTT) and 50 healthy controls.

Guinea pig skin test. Two groups of four random-bred Hartley female guinea pigs (Harlan Laboratories, Madison, WI, USA), each weighing ~ 300 g, were used for skin testing. One group was infected by aerosol with 10^2 *M. tuberculosis* $H_{37}Rv$ cells, and a second, control group was mock sensitized by intradermal injection of phosphate-buffered saline (PBS). Six weeks after infection, animals were intradermally injected with 1 μg of PPD and with 2 μg of each purified antigen on the shaved back. Injection sites were located 2.5 cm from each other. Diameters (in mm) of skin reaction (induration plus erythema) were measured 24 h after antigen injection.

Enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates were coated with antigen in 0.1 M carbonate-bicarbonate buffer pH 9.6 at 4 °C overnight. Coating antigen concentrations ranged between 0.1 μg/ml to 3 μg/ml. Guinea pig sera were used at a 1 : 50 dilution in PBS plus 0.05% Tween 20 (PBS-T). Immunodetection was performed with a rabbit antiguinea-pig immunoglobulin (Ig)G antibody conjugated with alkaline phosphatase (Sigma, St Louis, MD, USA) and an alkaline phosphatase substrate kit (Bio-Rad, Hercules, CA, USA). Optical density was measured at 405 nm (OD_{405}) with a microtiter plate reader (Spectra Shell, Tecan, Salzburg, Austria).

Multi-antigen print immunoassay (MAPIA). IgG antibodies against RD1 antigens in human sera were detected by MAPIA, a method that utilizes nitrocellulose membranes as solid phase for antigen coating [15]. Briefly, proteins were immobilized on nitrocellulose membranes (Protran BA83, Schleicher & Schuell, Dassel, Germany) as narrow bands by using a semiautomatic air-brush printing device (Linomat IV, Camag, Muttenz, Switzerland). Coating antigen concentrations ranged from 50 μg/ml to 200 μg/ml in PBS. The antigen-coated nitrocellulose sheet was cut into 4-mm wide strips, which were blocked for 1 h in 1% nonfat skim milk in PBS-T and then incubated for 1 h with serum samples diluted 1 : 50 in the same blocking buffer. After washing with PBS-T, strips were incubated for 1 h with alkaline-phosphatase-labelled antihuman IgG antibody (Sigma) diluted 1 : 2000 in PBS-T, and washed again with PBS-T. Enzyme activity was visualized by incubating strips for 10 min with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MA, USA). Strips were rinsed in distilled water to stop the reaction. The entire procedure was performed at room temperature. The presence of a visible band was scored as positive.

RESULTS AND DISCUSSION

RD1 is a 9.5 kilobase segment of the *M. tuberculosis* genome that is absent from the DNA of all substrains of *M. bovis* BCG [9]. In earlier work by Ahmad *et al.* [16], the products of three open reading frames in RD1 were expressed and serologically characterized [one partly corresponding to Rv3877 and two small ones that are not predicted by the sequence analysis of Cole *et al.* [5]. We focused our present analyses on the products of six other open reading frames in the RD1 region [Rv3871, Rv3872, Rv3873, MTSA-10 [13], ESAT-6 [17], and Rv3878] identified by the genome sequence analysis of Cole *et al.* [5]. Proteins were purified to near-homogeneity from recombinant *E. coli* (data not shown) and analyzed for the ability to elicit delayed type hypersensitivity (DTH) and antibody responses in tuberculous guinea pigs. Results of guinea pig skin testing are shown in Fig. 1. Of the antigens, two, ESAT-6 and MTSA-10 [also called CFP10 [18]], were already known to elicit TB-specific skin reactions in guinea pigs [13, 19, 20]. As previously shown [13], recognition of MTSA-10 varied broadly from animal to animal (from 0 mm to 14 mm diameter of skin reaction; Fig. 1). Of the four remaining RD1 antigens, three (the products of genes Rv3871, Rv3872, and Rv3878) elicited no DTH response and one (the Rv3873 product) gave only a low-level (2–4 mm) skin reaction in tuberculous animals (Fig. 1). Results of guinea pig serological analysis are shown in Fig. 2. Tuberculous animals showed a vigorous antibody response to all six RD1 antigens. Antibody levels to each antigen varied from animal to animal, with some animals having no antibodies to certain antigens (e.g. 3 of 13 in the case of ESAT-6 and 4 of 13 in the case of Rv3878). Because bacillary loads tend to be very

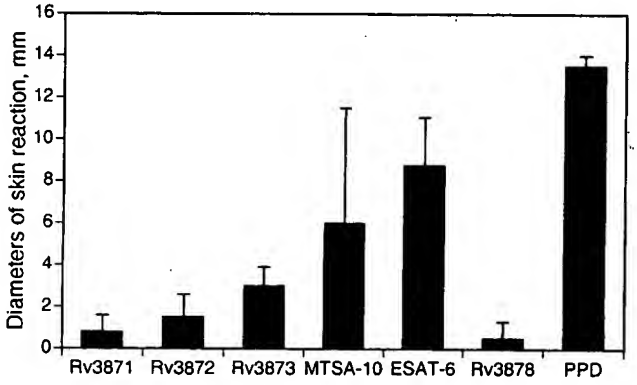

Fig. 1. Delayed-type hypersensitivity (DTH) to RD1 antigens in tuberculous guinea pigs. Two groups of four guinea pigs were used for skin testing. One group was aerosol-infected with *M. tuberculosis* $H_{37}Rv$, and a second, control group was mock sensitized by intradermal injection of phosphate-buffered saline (PBS) (no skin test reaction, not shown). Six weeks after infection, animals were intradermally injected with 1 μg of PPD and with 2 μg of each of the indicated purified antigens. Diameters (in mm) of skin reaction (induration plus erythema) were measured 24 h after antigen injection. The large standard deviation shown for MTSA-10 is owing to the broad animal-to-animal variation in the recognition of this antigen [13]. Results obtained with Rv3873 were normalized against the saline controls, because this protein tended to give 2–3 mm background reactions in saline control animals.

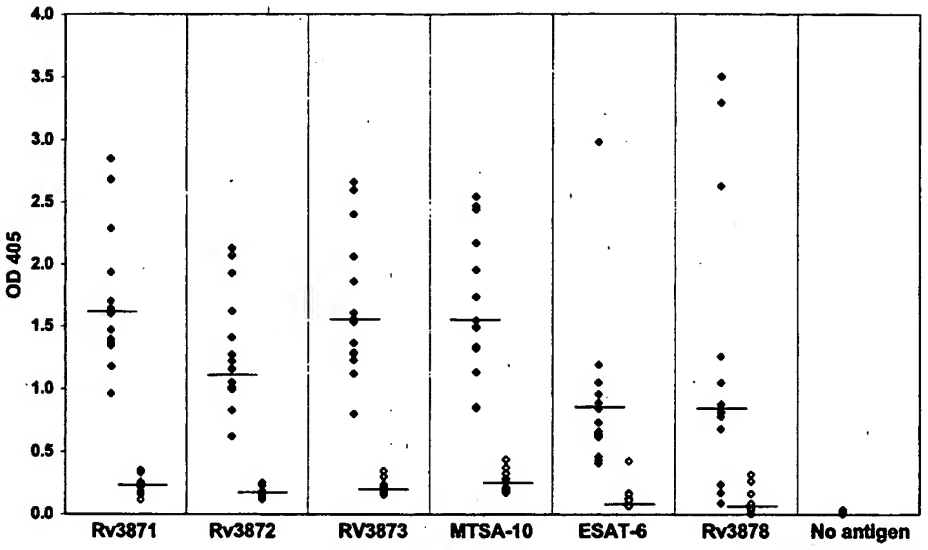

Fig. 2. Antibody responses to RD1 antigens in guinea pigs. Levels of serum IgG antibodies were analyzed by ELISA in 14 guinea pigs aerosol-infected with *M. tuberculosis* $H_{37}Rv$ (◆) and in 9 uninfected, control animals (◇). Each data point represents one animal. Horizontal bars indicate median values. Nine sera that reacted strongly with multiple antigens were assayed in mock-coated plates to control for nonspecific binding ('no antigen').

uniform in guinea pigs infected with low-dose aerosol [21], the observed heterogeneity in immune responses should be mainly attributable to the major histocompatibility complex (MHC) haplotype diversity found in random-bred animals [22, 23].

The observation that only two of six RD1 proteins induced strong DTH in tuberculous animals merits further comment. In general, it is not surprising that only a small proportion of proteins selected using a genome-based approach turns out to possess the activity under investigation. However, the guinea pig skin test results may underestimate DTH activity of the RD1 antigens for either of two reasons. First, the skin test may not be sensitive enough to detect low-level T-cell responses. Indeed, T-cell proliferative responses to some antigens can be detected under conditions in which a positive skin test is not elicited by those antigens (D.N. McMurray, unpublished observations). Secondly, some antigens of *M. tuberculosis* express DTH activity only after undergoing post-translational modification such as glycosylation or acylation [24, 25], and such modification does not occur in *E. coli*. The modification state of the RD1 antigens in *M. tuberculosis* cells is not known. Thus, the absence of post-translational modification may account for the observed lack of DTH activity for some of the RD1 proteins expressed in *E. coli*.

Because all RD1 antigens elicited antibody responses in guinea pigs, we next assessed their serological activity in human TB (Table 1). Sera were obtained from 75 patients having culture-confirmed pulmonary TB, 16 patients having culture-confirmed MOTT disease, and 50 healthy blood donors. Antibody detection was conducted by MAPIA, a nitrocellulose membrane-based assay described in the Materials and Methods. Representative results are presented in Fig. 3. The Rv3873 protein tended to give background reactions with negative control sera (data not shown) and was excluded from further evaluation. No antibodies to the remaining five antigens (Rv3871, Rv3872, MTSA-10, ESAT-6, and Rv3878) were detected in sera from 66 control subjects (Table 1). The absence of cross-reactivity of these antigens with sera from MOTT patients is consistent with the observed lack of homologs for these proteins in *M. avium*, which is the commonest cause of non-TB mycobacterioses in humans [8]. In fact, amino acid sequence identity between the five RD1 proteins and *M. avium* proteins was low (ranging from 24% to 35%), with identical amino acid residues scattered throughout the sequences [BLAST homology data utilizing a partial genome sequence of *M. avium* (http://www.tigr.org/tdb/); not shown]. Among TB patients, only a few (3% to 7%) responded to Rv3871, Rv3872 and Rv3878 proteins, while a sizable proportion had antibodies against ESAT-6 (13%) and MTSA-10 (16%) (Table 1). Together, responders to ESAT-6 and/or MTSA-10 accounted for 25% of the TB patient cohort (data not shown). The present findings are consistent with our previous work on serological reactivity to ESAT-6 in human TB [4] and with further serological

Table 1. Antibody responses to RD1 antigens in human subjects

Antibody responders among	Rv3871	Rv3872	MTSA-10	ESAT-6	Rv3878
	(%)	(%)	(%)	(%)	(%)
TB patients	3/75 (4)	2/75 (3)	12/75 (16)	10/75 (13)	5/75 (7)
MOTT patients	0/16	0/16	0/16	0/16	0/16
Healthy controls	0/50	0/50	0/50	0/50	0/50

The table shows number and percentage of antibody responders to RD1 antigens among 75 patients having active pulmonary TB, 16 patients with non-TB mycobacterioses (MOTT), and 50 healthy controls. Serum IgG antibodies were detected by MAPIA (representative results are shown in Fig. 3). Ten of 16 sera from MOTT patients reacted strongly with *M. tuberculosis* culture filtrates (data not shown), indicating that these patients had mounted an antibody response to mycobacterial infection.

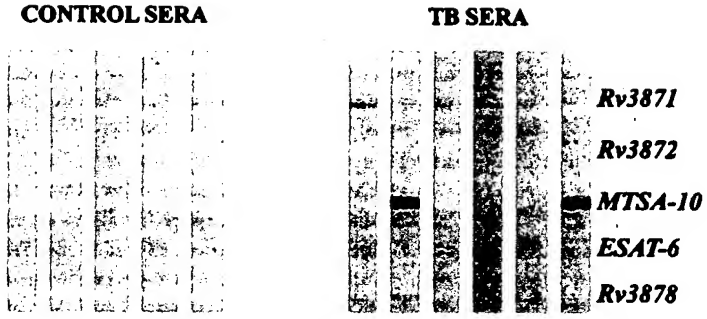

Fig. 3. Detection of antibody to RD1 antigens in human sera. Human serum IgG antibodies to antigens immobilized on nitrocellulose membranes were detected by MAPIA (see Materials and Methods). Images are shown of representative strips printed with five RD1 antigens (as indicated) and developed after incubation with sera from five healthy individuals (negative control sera) and five tuberculosis patients (TB sera). Strip images were obtained with a Hewlett Packard ScanJet IIcx/T scanner.

evaluation of MTSA-10 using larger sets of sera from TB patients and controls (M.L. Gennaro *et al.* unpublished data). The serodiagnostic potential of MTSA-10 has also been recently recognized by Dillon *et al.* [26].

Our data on the Rv3873 protein enable us to comment on its immunodiagnostic potential. The Rv3873 protein is one of 68 PPE proteins of *M. tuberculosis*. These acidic, glycine-rich proteins are characterized by a conserved Pro-Pro-Glu sequence at their NH_2-terminus [5], they share extensive sequence homology with each other [5], and they may have homologs in nontuberculous mycobacteria. For example, the Rv3873 protein shares 50% identity with a protein of *M. avium*, as indicated by a BLAST homology search that used a partial genome sequence of *M. avium* (http://www.tigr.org/tdb/) (data not shown). The observations that Rv3873 elicits a low-level DTH response (2–4 mm) in tuberculous guinea pigs, 2–3 mm background reactions in control, mock-infected guinea pigs, and elevated background reactivity in control human sera suggest the presence of cross-reactive sequences in this protein.

In conclusion, the work reported here defines some of the immunological properties of six antigens encoded by the RD1 region of the M. *tuberculosis* genome. The products of *rv3871*, *rv3872*, and *rv3878* appear to have limited immunodiagnostic potential because they elicit no DTH in tuberculous guinea pigs and are recognized by serum antibodies of only a small proportion of patients having active TB. The product of *rv3873*, a PPE protein, induces measurable, but nonspecific, immune responses both in experimental and human TB. Finally, ESAT-6 and MTSA-10 are recognized by a substantial proportion of sera from TB patients and are expected to contribute few, if any, false-positive reactions. Thus, the latter two are ideal components of a multiantigen serodiagnostic cocktail.

ACKNOWLEDGMENTS

We wish to thank staff at the Painter Center at CSU (Alan Roberts for aerosol infection of guinea pigs, Elisa French and Julia Granowski for animal care); John Belisle for advice on guinea pig skin testing; Karl Drlica for comments on the manuscript. R.C. was the recipient of a fellowship on tuberculosis research from the Istituto Superiore di Sanita', Rome, Italy. X.Z. was a student at the Stuyvesant High School in New York, NY (USA) named semifinalist of the Intel Science Talent Search for her contribution to the present work. This work was supported by NIH grant AI-36989 (M.L.G.) and by NIH/NIAID contract NO1-AI-75320 'Tuberculosis Research Materials and Vaccine Testing'. This paper is publication no. 76 from the PHRI TB Center.

REFERENCES

1 Gennaro ML. Immunologic diagnosis of tuberculosis. Clin Infect Dis 2000;30:S243–6.
2 Shinnick TM. Diagnostic test needs for evaluating antituberculosis vaccines. Clin Infect Dis 2000;30:S276–8.
3 Schoel B, Gulle H, Kaufmann SHE. Heterogeneity of the repertoire of T cells of tuberculosis patients and healthy contacts to *Mycobacterium tuberculosis* antigens separated by high-resolution techniques. Infect Immun 1992;60:1717–20.
4 Lyashchenko K, Colangeli R, Houde M, Jahdali HA, Menzies D, Gennaro ML. Heterogeneous antibody responses in tuberculosis. Infect Immun 1998;66:3936–40.
5 Cole ST, Brosch R, Parkhill J *et al.* Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 1998;393:537–44.
6 Behr MA, Wilson MA, Gill WP *et al.* Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 1999;284:1520–3.
7 Gordon SV, Brosch R, Billault A, Garnier T, Eiglmeier K, Cole ST. Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. Mol Microbiol 1999;32:643–55.
8 Grange J. Other mycobacterial diseases. In: Grange, J ed. Mycobacteria and Human Disease. London, UK: Arnold, 1996:188–203.
9 Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J Bacteriol 1996;178:1274–82.
10 Manca C, Lyashchenko K, Colangeli R, Gennaro ML. MTC28, a novel 28-Kilodalton proline-rich secreted antigen specific for the *Mycobacterium tuberculosis* complex. Infect Immun 1997;65:4951–7.
11 Colangeli R, Heijbel A, Williams A *et al.* Three-step purification of lipopolysaccharide-free, polyhistidine-tagged recombinant antigens of *Mycobacterium tuberculosis*. J Chromatogr B 1998;714:223–35.
12 Lyashchenko K, Manca C, Colangeli R, Heijbel A, Williams A, Gennaro ML. Use of *Mycobacterium tuberculosis* complex-specific

antigen cocktails for a skin test specific for tuberculosis. Infect Immun 1998;66:3606–10.

13 Colangeli R, Spencer JS, Bifani P *et al.* MTSA-10, the product of the Rv3874 gene of *Mycobacterium tuberculosis*, elicits tuberculosis-specific, delayed-type hypersensitivity in guinea pigs. Infect Immun 2000;68:990–3.

14 Wiegeshaus EH, McMurray DN, Grover AA, Harding GE, Smith DW. Host-parasite relationships in experimental airborne tuberculosis. III. Relevance of microbial enumeration to acquired resistance in guinea pigs. Am Rev Resp Dis 1970;102:422–9.

15 Lyashchenko KP, Singh M, Colangeli R, Gennaro ML. A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. J Immunol Meth 2000;242:91–100.

16 Ahmad S, Amoudy HA, Thole JE, Young DB, Mustafa AS. Identification of a novel protein antigen encoded by a *Mycobacterium tuberculosis*-specific RD1 region gene. Scand J Immunol 1999;49:515–22.

17 Sørensen AL, Nagai S, Houen G, Andersen P, Andersen ÅB. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. Infect Immun 1995;63:1710–7.

18 Berthet F-X, Rasmussen PB, Rosenkrands I, Andersen P, Gicquel B. A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology 1998;144:3195–203.

19 Elhay MJ, Oettinger T, Andersen P. Delayed-type hypersensitivity responses to ESAT-6 and MPT64 from *Mycobacterium tuberculosis* in the guinea pig. Infect Immun 1998;66:3454–6.

20 van Pinxteren LA, Ravn P, Agger EM, Pollock J, Andersen P. Diagnosis of Tuberculosis Based on the Two Specific Antigens ESAT-6 and CFP10. Clin Diagn Lab Immunol 2000;7:155–60.

21 McMurray DN, Mintzer CL, Tetzlaff CL, Carlomagno MA. The influence of dietary protein on the protective effect of BCG in guinea pigs. Tubercle 1986;67:31–9.

22 Haslov K, Andersen AB, Ljungqvist L, Weis Bentzon M. Comparison of the immunological activity of five defined antigens from *Mycobacterium tuberculosis* in seven inbred guinea pig strains. The 38 kDa antigen is immunodominant. Scand J Immunol 1990;31:503–14.

23 Mackall JC, Bai GH, Rouse DA *et al.* A comparison of the T cell delayed-type hypersensitivity epitopes of the 19-kD antigens from *Mycobacterium tuberculosis* and *Mycobacterium intracellulare* using overlapping synthetic peptides. Clin Exp Immunol 1993;93:172–7.

24 Horn C, Namane A, Pescher P *et al.* Decreased capacity of recombinant 45/47-kDa molecules (Apa) of *Mycobacterium tuberculosis* to stimulate T lymphocyte responses related to changes in their mannosylation pattern. J Biol Chem 1999;274:32023–30.

25 Romain F, Horn C, Pescher P *et al.* Deglycosylation of the 45/47-kilodalton antigen complex of *Mycobacterium tuberculosis* decreases its capacity to elicit *in vivo* or *in vitro* cellular immune responses. Infect Immun 1999;67:5567–72.

26 Dillon DC, Alderson MR, Day CH *et al.* Molecular and immunological characterization of mycobacterium tuberculosis CFP-10, an immunodiagnostic antigen missing in *Mycobacterium bovis* BCG. J Clin Microbiol 2000;38:3285–90.